Genetic control of neuronal activity in mice conditionally expressing *TRPV1*

Benjamin R Arenkiel, Marguerita E Klein, Ian G Davison, Lawrence C Katz & Michael D Ehlers

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Supplementary Methods

Note: Supplementary Movies 1–3 are available on the Nature Methods website.

Genetic Control of Neuronal Activity in Mice Conditionally Expressing TRPV1

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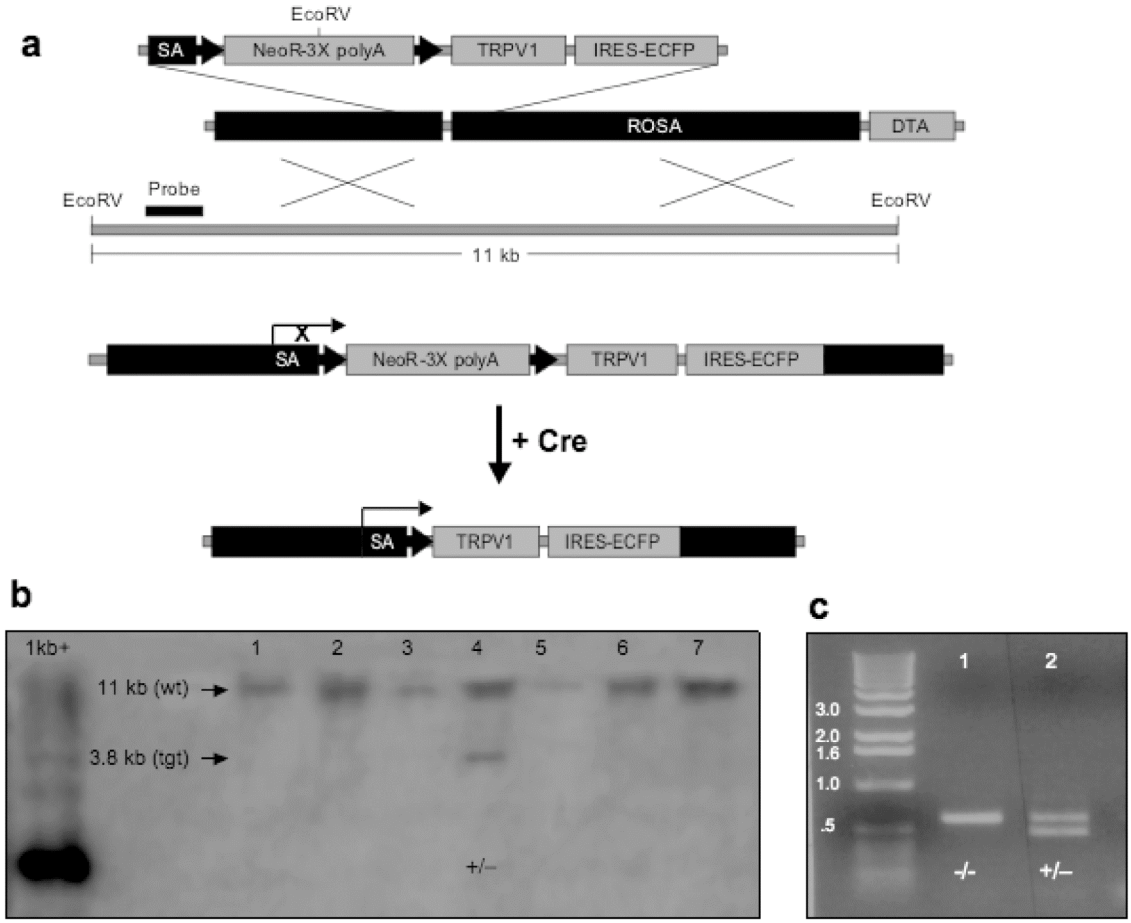
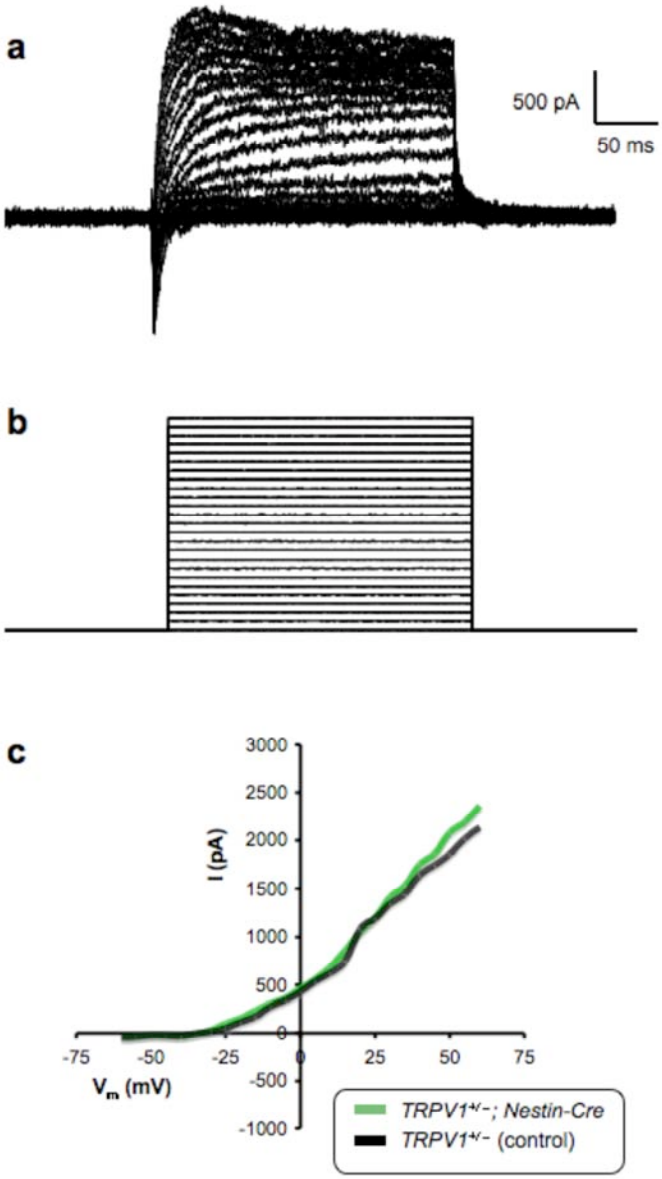
SUPPLEMENTARY MATERIAL

Supplementary Figure 1



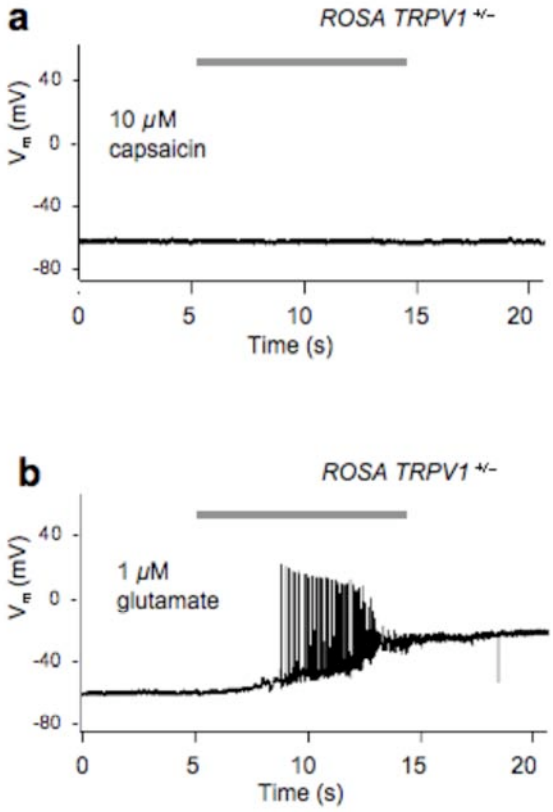
Supplementary Figure 1. Gene targeting of the conditional *TRPV1-IRES-ECFP* sequence to the *ROSA26* locus by homologous recombination. (**a**) Diagram of the conditional targeting vector and the expected recombination events in the presence of Cre recombinase. *SA*, splice acceptor sequence; thick arrowheads, *loxP* sites; *NeoR*, neomycin resistance positive selection cassette; *DTA*, diphtheria toxin negative selection cassette. (**b**) Southern blot analysis showing the identification of positively targeted ES cell clones after digestion with EcoRV and hybridization with the probe region shown in (a). A positive targeting event is shown in lane 4. (**c**) PCR genotyping of mouse pups to identify offspring harboring the conditional allele. Ladder in the left lane is in kilobases.

Supplementary Figure 2



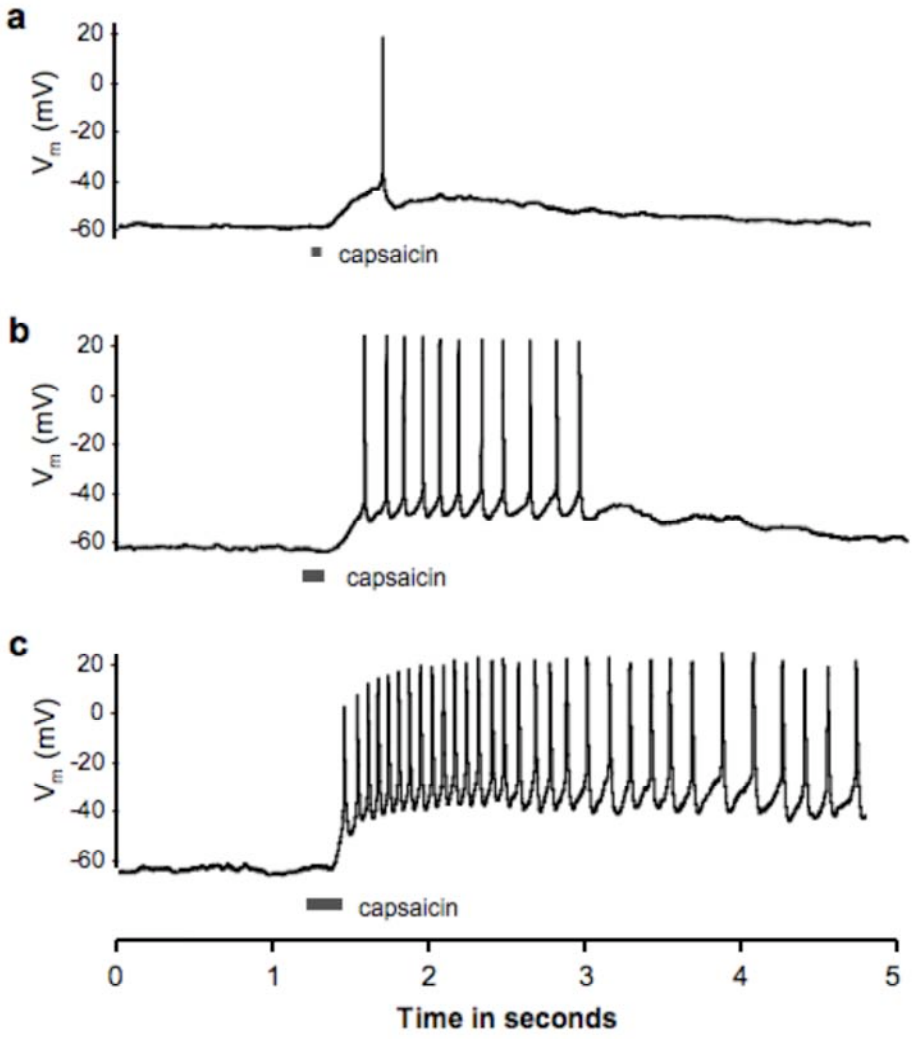
Supplementary Figure 2. TRPV1-expressing neurons elicit normal passive electrophysiological responses. (**a**) An example of potassium current responses recorded from a TRPV1 expressing neuron recombined by Cre in culture to 5 mV steps in membrane potential. (**b**) Corresponding 5 mV stepwise voltage changes used to induce the currents shown in (a). Time scale as in (a). (**c**) Example current-voltage (I-V) curves from TRPV1-expressing (green trace) and control, non-recombined neurons (black trace).

Supplementary Figure 3



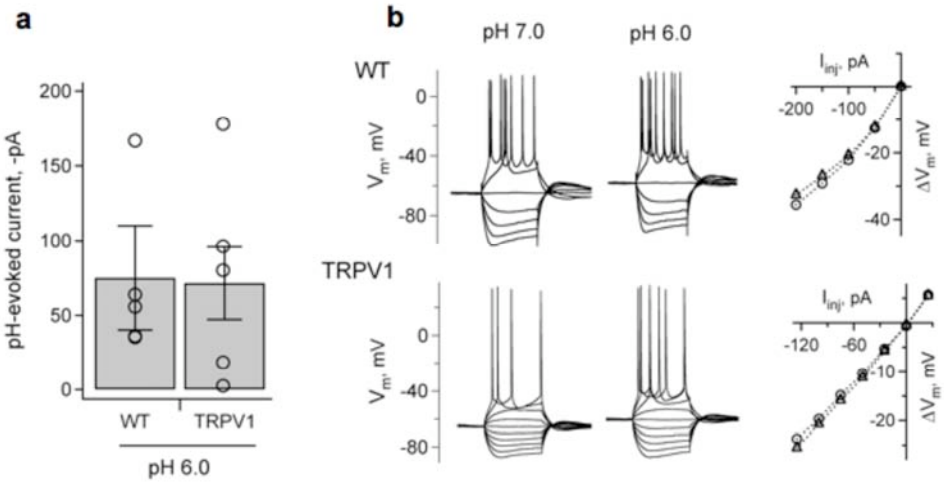
Supplementary Figure 3. Neocortical neurons in unrecombined control slices lack capsaicin-evoked responses. (**a**) Control cells from *ROSA-stop^{flox}-TRPV1-IRES-ECFP* animals (*ROSA TRPV1 +/-)* not expressing the conditional TRPV1 reporter due to the absence of Cre recombinase do not show capsaicin-mediated currents. Note the higher concentration of capsaicin relative to that used in **Fig. 2e**. (**b**) Control cells not expressing TRPV1 still respond to glutamate. Recordings are from neocortical neurons in brain slices from unrecombined *ROSA-stop^{flox}-TRPV1-IRES-ECFP* animals (*ROSA TRPV1 +/-)* as in (a). The gray bars in (a) and (b) indicate the time of capsaicin or glutamate application, respectively. Recordings in (a) and (b) were performed in the absence of pharmacological blockers of fast synaptic transmission.

Supplementary Figure 4



Supplementary Figure 4. Local application of 1 μM capsaicin for different durations on TRPV1 expressing neurons induces neuronal firing in a graded manner. Traces shown in (**a**-**c**) correspond to the differential responses from a neuron in a slice from a *nestin-Cre::ROSA-stop^{flox}-TRPV1-IRES-ECFP* mouse following application of capsaicin for different periods of time. **(a)** Brief application of capsaicin (50 ms) can elicit single spikes. **(b)** Longer duration application of capsaicin (100 ms) elicits reproducible and recoverable spike trains. **(c)** Prolonged capsaicin (200 ms) induces sustained high frequency firing that desensitizes with time.

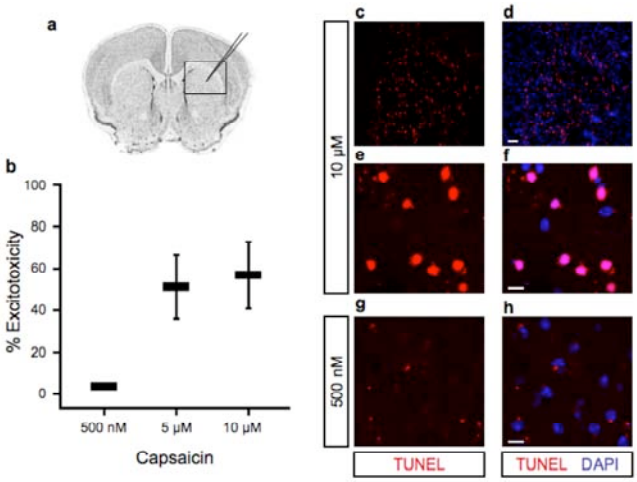
Supplementary Figure 5



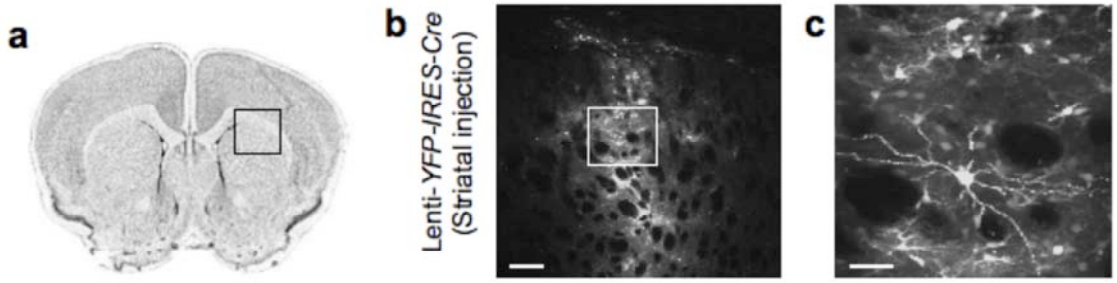
Supplementary Figure 5. Neurons in TRPV1-expressing mice exhibited no change in pH sensitivity within physiological ranges. (**a**) Currents evoked in WT and TRPV1-expressing cortical pyramidal neurons by switching from pH 7.4 bicarbonate-buffered ACSF to pH 6.0 HEPES-buffered ACSF. The magnitudes of pH-evoked currents were indistinguishable between WT and TRPV1 expressing neurons . Cells were voltage-clamped at -70 mV. n = 5, p > 0.9. Error bars indicate SEM. (**b**) Responses to current injection in pyramidal neurons in brain slices derived from WT (top) and TRPV1 expressing mice (bottom), tested at pH 7.0 and pH 6.0. Lowering the pH produced a small depolarization in both cases, but TRPV1 expression did not change the excitability or input resistance at different pH levels. Left panels, example voltage traces for current injections ranging from -125 to 50 pA for the wildtype (top), or -250 to 150 pA for the TRPV1 expressing (bottom) upon a switch to pH 7.0 or pH 6.0. Right panels, current (I_{inj})-voltage (ΔV_m) relationships from wt and TRPV1-expressing neurons upon a switch to pH 7.4 (circles) or pH 6.0 (triangles).

Supplementary Figure 6

Supplementary Figure 6. Applying high concentrations of capsaicin induces excitotoxic cell death of recombined neurons in *nestin-Cre::ROSA-stop^{flox}-TRPV1-IRES-ECFP* mice. **(a)** Coronal section through an adult mouse brain highlighting the striatal domain (inset) targeted for capsaicin infusion. **(b)** Graph illustrating the relative levels of cell death observed in the striatum following infusion of different concentrations of capsaicin. Data represent means ± SEM. n = 12 independent section counts from two animals at each concentration. **(c)** Apoptotic (TUNEL-positive) striatal neurons observed following infusion of 10 µM capsaicin. **(d)** DAPI overlay of (b). **(e)** High magnification view of apoptosis of striatal neurons in response to 10 µM capsaicin infusion. **(f)** DAPI overlay of (e). **(g)** High magnification view of striatal neurons lacking TUNEL stain following infusion of 500 nM capsaicin. **(h)** DAPI overlay of (g). Scale bars: (c,d), 70 µm; (e-h), 10 µm.



Supplementary Figure 7



Supplementary Figure 7. Focal infection of striatal neurons with a *YFP-IRES-Cre* expressing lentivirus. (**a**) Coronal section of mouse brain illustrating the striatal region (shown as inset) that was targeted for *YFP-IRES-Cre* lentivirus injection into *ROSA-stop^{flox}-TRPV1-IRES-ECFP* conditional mice. (**b**) Focal expression of YFP-IRES-Cre in striatal neurons. Fluorescent signal indicates YFP fluorescence. Scale bar, 50 μm. (**c**) High magnification of the inset shown in (b). Scale bar, 25 μm. See **Supplementary Movie 3** for capsaicin-evoked turning response in mice focally expressing TRPV1.

Supplementary Movie 1. Capsaicin-mediated neuronal activation modifies behavior in awake behaving mice conditionally expressing TRPV1. Shown is the behavioral response of a *nestin-Cre::ROSA-stop^{flox}-TRPV1-IRES-ECFP* mouse that was infused with 500 nM capsaicin via a cannula implanted in the dorsal left striatum. The movie shows representative activity prior to, 5 min after, and 15 min after capsaicin treatment. Mice fully recovered by 15 min post-infusion.

Supplementary Movie 2. Capsaicin infusion does not affect locomotor behavior in control mice. Shown is the normal locomotor behavior before and after capsaicin infusion in *ROSA-stop^{flox}-TRPV1-IRES-ECFP* control animals lacking Cre expression.

Supplementary Movie 3. Focal expression of TRPV1 unilaterally in the striatum results in contralateral circling behavior upon infusion with capsaicin. Shown is the behavioral response from a *ROSA-stop^{flox}-TRPV1-IRES-ECFP* mouse locally infected in the right striatum with a *YFP-IRES-Cre* expressing lentivirus following infusion with 500 nM capsaicin 1 week post-infection.

Supplementary Methods

Generation of conditional "knock-in" mice

The *ROSA26* genomic DNA used for targeting vector design was obtained from F. Costantini[1] and modified by replacing the fluorescent reporter sequence with the rat *TRPV1* cDNA[2] followed by an *IRES-ECFP* sequence. The final targeting vector was linearized with PvuI, electroporated into SVJ129 ES cells, and selected on G418 (200 μg/ml, 6 days). Positive ES clones were identified by Southern blotting using a 0.5 kb, 5' external probe on EcoRV digested genomic DNA. Positive ES clones were subsequently injected into Bl6 blastocysts and germline transformants verified by multiplexed PCR genotyping (ROSA 5', TCCCAAAGTCGCTCTGAGTT; ROSA 3', ACTCGGGTGAGCATGTCTTT; ECFP, GCATGGACGAGCTGTACAAG) and Southern analysis.

Biolistic introduction of Cre recombinase

Brains from postnatal day 14 mice were dissected and coronal slices through the main olfactory bulb were cut at a thickness of 300 μm. Gold particles (1 μm) were coated with a *CMV-Cre* plasmid and accelerated into slices using a Helios gene gun (Biorad, Hercules, CA). Slices were cultured for 3 days and subsequently analyzed for gene expression.

Lentivirus engineering, production and neuronal infection

Standard molecular cloning methods were used to replace the *ubiquitin-EGFP* of *FUGW* with *CMV YFP-IRES-Cre*[3]. Lentivirus was made by transfecting $6x10^6$ 293FT (Invitrogen, Carlsbad, CA) cells with 5 μg VSVg, 15 μg Δ8.9 and 20 μg promoter-reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72 hours, supernatants were harvested, filtered through 0.45 μm syringe filters (Corning, Corning, NY), and pelleted by ultracentrifugation at 26,000 rpm for 2 hours at 4ºC. Following resuspension in HBSS, lentivirus was stored at -80ºC. For infections, lentivirus was added to culture media overnight and washed off the next day. Reporter expression was analyzed 72 hours or more post-infection.

Confocal imaging and immunohistochemistry

Nestin-Cre::ROSA-stop^{flox}-TRPV1-IRES-ECFP animals were humanely sacrificed in accordance with Duke University IACUC procedures, perfused with 4% paraformaldehyde in phosphate buffered saline, and post-fixed for 1 hour at 4˚C. Brain tissue was then embedded in O.C.T and sectioned to 10 μm on an upright Leica cryostat. Sliced tissues or coverslipped cultures were mounted on slides and imaged using a Zeiss 510 scanning confocal microscope (Carl Zeiss Inc.). For immunohistochemistry, sections or cultured neurons were incubated with blocking solution (10% normal goat serum, 2% BSA, 0.1% Triton X-100 in PBS pH 7.4) and incubated at 4˚C for 2 hrs. Monoclonal anti-GFP, 1:1000 (Molecular Probes Inc., Eugene, OR) or rabbit polyclonal anti-TRPV1, 1:1000 (Chemicon, Temecula, CA) antibodies were diluted in blocking solution and applied overnight at 4˚C. The next day, sections were washed 3 x 15 min each in PBS containing 0.1% Triton, followed by 2 x 15 min in blocking solution. Secondary Alexa-488-conjugated goat anti-mouse IgG and Alexa-555-conjugated goat anti-rabbit (Molecular Probes Inc., Eugene, OR) were then added to a final dilution of 1:500 and incubated for 4 hrs at 4˚C. Slices were then washed 4 x 15 min each and mounted with DAPI-containing Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Cell culture electrophysiology

Primary cortical cultures were made from P1 *ROSA-stop^{flox}-TRPV1-IRES-ECFP* conditional mice as described[4]. Whole-cell voltage clamp recordings were performed on DIV 10 – 15 cortical neurons cultured at high density on poly-lysine coated glass coverslips. Capsaicin-evoked currents were elicited by local pressure ejection of 1 μM capsaicin from a micropipette using a FemtoJet (Eppendorf, Westbury, NY) and recorded using a MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) controlled with a Pentium PC running MultiClamp Commander and pClamp (Axon Instruments). The extracellular solution contained: 150 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 30 mM *D*-glucose, 2 mM $CaCl_2$, 1.0 μM TTX, 30 μM bicuculline (330 mOsm/l, pH 7.4). Recording pipettes, with resistances between 3-5 MΩ, were filled with a solution containing: 30 mM $CsSO_4$, 70 mM K_2SO_4, 25 mM HEPES, 25 mM *N*-methyl-*D*-

glucamine, 0.1 mM $CaCl_2$, 1 mM EGTA, 2 mM Na_2ATP, 0.1 mM leupeptin (300 mOsm/l, pH 7.2).

Slice electrophysiology

Whole brains from three week old mice were dissected into ice cold ACSF. Slices (350 μm) were cut in ice cold ACSF containing sucrose (87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 75 mM sucrose, 10 mM glucose, 1.3 mM ascorbic acid, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$) and incubated at 30˚C for 15 min in CO_2-buffered ACSF (124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 20 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$) followed by room temperature incubation for visually guided patch recordings. Patch pipettes were pulled on a Model P-97 micropipette puller (Sutter Instruments Inc., Novato, CA) to a tip resistance of 3.5 – 7 MΩ. The internal electrode solution contained 130 mM K gluconate, 10 mM HEPES pH 7.4, 2 mM $MgCl_2$, 2 mM MgATP, 0.5 mM NaGTP, 1 mM EGTA, 10 mM phosophocreatine. Neuronal sensitivity to pH was tested in two ways (**Supplementary Fig. 5).** Under voltage clamp, neurons were held at -70 mV, and rapid perfusion of normal ACSF was replaced with HEPES-buffered ACSF at pH 6.0 for 90-120 sec. ACSF exchange produced a change in holding current at both pH levels, likely due to differences in ionic composition and oxygenation level in addition to shifts in pH. However, there was no difference in responses of WT and TRPV1 neurons. Cells were also recorded in current clamp mode, and tested for their response to a range of current injections at either pH 7.0 or pH 6.0 conditions. Although reducing pH from 7.0 to 6.0 produced a slight depolarization of both WT and TRPV1 neurons, ectopic TRPV1 expression did not change input resistance or excitability.

***In vivo* electrophysiology**

In vivo recordings were performed on animals anesthetized with injected ketamine and xylazine (150 μg ketamine and 6 μg xylazine per gram of body weight via i.p. injection), followed by sustained 0.5% isofluorane. The dorsal surface of the brain was exposed by a small window craniotomy. Electrophysiological recordings were made using 1 MΩ

extracellular recording electrodes (Microprobe Inc., Gaithersburg, MD) that were fixed to glass injection pipettes filled with 1 μM capsaicin attached to a pneumatic Picospritzer II (General Valve Corp., Fairfield, NJ). Recordings were band pass filtered at 300 Hz low / 5 kHz high using a model 1800 AC differential amplifier (A-M Systems, Carlsborg, WA). Data was collected and processed using Spike2 acquisition software (Cambridge Electronic Design, Cambridge, UK). PSTHs were assembled for each unit recorded by compiling both baseline and post-stimulus data from multiple injection trials.

***In vivo* behavioral manipulations in awake animals**

Canulas (ALZET, Cupertino, CA) were fixed to the skulls of mice anesthetized with injected ketamine and xylazine (150 μg ketamine and 6 μg xylazine per gram of body weight via i.p. injection), followed by sustained 0.5% isofluorane. All procedures were performed in accordance with guidelines of the Duke University Institutional Animal Care and Use Committee. The next day, ~ 2.5 μl of capsaicin (500 nM) was acutely infused into the canulas with a Hamilton syringe at a rate of 0.5 μl/sec. For regional expression of TRPV1 in the striatum of conditional animals, 2 μl of YFP-IRES-Cre lentivirus was injected. One week following viral injections, mice were infused with capsaicin as described above. Behavioral responses were digitally recorded with a camcorder and edited in Imovie (Apple).

***In vivo* cell death assays**

Nestin-Cre::ROSA-stop^{flox}-TRPV1-IRES-ECFP mice were anesthetized and stereotaxically injected with 2.0 μl of variable concentrations of capsaicin at a delivery rate of 46.0 nl/sec. At 24 hrs post-injection, brains were dissected and fixed in 4% PFA in PBS, pH 7.4. Fixed brains were sectioned at 20 μm, mounted on Superfrost plus slides, and reacted with an In Situ Cell Death Detection Kit, TMR red (Roche, Germany). Apoptotic neurons (TUNEL-positive) were counted in striatal tissue and referenced against DAPI nuclear staining. Percent excitotoxicity was determined from random 75 μm x 75 μm areas throughout the injection region. Graph values in **Supplementary Fig. 6b** were obtained from 12 independent section counts from two animals at each concentration. Error bars represent SEM.

References

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